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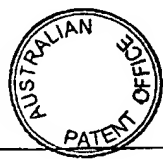
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(54) Title: PURIFICATION AND/OR CONCENTRATION OF DNA BY CROSS-FLOW FILTRATION, SEPARATION OF ENDOTOXINS FROM A NUCLEIC ACID PREPARATION			
(54) Bezeichnung: REINIGUNG ODER/UND KONZENTRIERUNG VON DNA DURCH CROSS-FLOW-FILTRATION, ABTRENNUNG VON ENDOTOXINEN AUS EINER NUCLEINSÄURE-PRÄPARATION			
(57) Abstract			
<p>The invention concerns a method of purifying and/or concentrating nucleic acids in a solution, the solution containing nucleic acid being guided tangentially past one or a plurality of semipermeable membranes, such that the nucleic acid molecules are retained by the membranes and substances having a lower molecular weight can pass through the membranes and/or be adsorbed thereat, so that a purified and/or concentrated nucleic acid solution is obtained. The same method is carried out to separate endotoxins from a nucleic acid preparation. The invention further concerns the use of a cross-flow filtration system for purifying and/or concentrating nucleic acids in a solution and for separating endotoxins from a nucleic acid preparation. The invention also concerns the use of the nucleic acids purified and/or concentrated by the cross-flow filtration system for cloning, transformation, transfection and microinjection into cells, for use in gene therapy processes, DNA vaccination and/or for polymerase chain reaction (PCR).</p>			
(57) Zusammenfassung			
<p>Die Erfindung betrifft ein Verfahren zur Reinigung oder/und zur Konzentrierung von Nucleinsäuren in einer Lösung, wobei man die Nucleinsäure enthaltende Lösung tangential an einer oder mehreren semipermeablen Membranen vorbeileitet, so daß die Nucleinsäure-Moleküle von den Membranen zurückgehalten werden und Substanzen mit einem geringeren Molekulargewicht durch die Membranen durchtreten können oder/und an der Membran adsorbiert werden und man eine gereinigte oder/und konzentrierte Nucleinsäure-Lösung erhält. In derselben Weise wird zur Abtrennung von Endotoxinen aus einer Nucleinsäure-Präparation verfahren. Weiter betrifft die Erfindung die Verwendung einer Cross-Flow-Filtrationsanlage zur Reinigung oder/und zur Konzentrierung von Nucleinsäuren in einer Lösung sowie zur Abtrennung von Endotoxinen aus einer Nucleinsäure-Präparation. Ferner die Verwendung der mit der Cross-Flow-Filtration gereinigten oder/und konzentrierten Nucleinsäuren zum Klonieren, zur Transformation, zur Transfektion, zur Mikroinjektion in Zellen, zur Verwendung bei Verfahren der Gentherapie, der DNA-Vakzinierung oder/und zur Polymerase-Ketten-Reaktion (PCR).</p>			

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Abstract

The invention concerns a method for purifying or/and concentrating nucleic acids in a solution, the solution containing nucleic acid being guided tangentially past over one or several semipermeable membranes such that the nucleic acid molecules are retained by the membranes and substances with a lower molecular weight can pass through the membranes or/and are adsorbed to the membrane to obtain a purified and/or concentrated nucleic acid solution. The same method is carried out to separate endotoxins from a nucleic acid preparation. In addition the invention concerns the use of a cross-flow filtration system to purify or/and concentrate nucleic acids in a solution and to separate endotoxins from a nucleic acid preparation. The invention also concerns the use of the nucleic acids purified and/or concentrated by cross-flow filtration for cloning, for transformation, for transfection, for microinjection into cells, for use in gene therapy methods, DNA vaccination or/and for the polymerase chain reaction (PCR).

PURIFICATION OR/AND CONCENTRATION OF DNA BY CROSS-FLOW
FILTRATION

Description

The present invention concerns a method for purifying
or/and concentrating nucleic acids in a solution.

Nucleic acid purification methods are common methods in
the field of molecular biology. In methods known from
the prior art the isolated biological material, such as
E. coli bacterial cells is for example centrifuged after
they have been lysed (usually lysis with lysozyme or
ultrasound) and the supernatant is shaken out with
phenol. Subsequently an ultracentrifugation is carried
out on a caesium chloride gradient (Birnboim & Doly,
Nucl. Acid Res. 7 (1979) 1513-1523; Garger et al.,
Biochem. Biophys. Res. Comm. 117 (1983) 835-842).



for the toxic effect of endotoxins.

Another method for purifying nucleic acids is described in the QIAGEN® Plasmid Handbook (Qiagen Inc., Chatsworth, USA) and in EP-B 0 268 946. According to this the cell lysate obtained by the usual lysis is chromatographed on a QIAGEN® TIP which contains QIAGEN® resin (a support material based on silica gel). A disadvantage of this method is that DNA binding proteins are not completely detached from the DNA so that the plasmid preparation that is obtained contains a considerable amount of proteins and in particular endotoxins (e.g. from the membrane of the E. coli cell).

In another nucleic acid purification method after alkaline lysis of the biological material for example E. coli cells the centrifugation supernatant is chromatographed according to Birnboim & Doly under high salt conditions over anion exchange columns (e.g. Mono-Q, Source-Q from Pharmacia, Macroprep-Q from Biorad, Poros-Q from Perspective Biosystems or HyperD-Q from Biosepra, cf. Chandra et al., *Analyt. Biochem.* 203 (1992) 169-172; Dion et al., *J.Chrom.* 535 (1990) 127-147). Even after this purification step the plasmid preparation still contains impurities such as proteins and especially a considerable amount of endotoxins.

In yet another method for purifying nucleic acids a chromatography is carried out by gel filtration after alkaline lysis and subsequent phenol-chloroform extraction (McClung & Gonzales, *Anal.Biochem.* 177 (1989) 378-382; Raymond et al., *Analyt.Biochem.* 173 (1988) 124-133). This purification method is also not able to completely remove the impurities from the plasmid

preparation.

The said purification methods all have a final desalting and concentration step. This usually involves an isopropanol/ethanol precipitation of the nucleic acid with subsequent centrifugation and resuspension of the nucleic acid pellet in buffer (cf. e.g. Sambrook J. et al. (1989), Molecular Cloning; A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press). In this process a DNA solution is for example admixed with 1/10 volumes 4 M LiCl and subsequent with 0.7 volumes isopropanol at room temperature. Subsequently the precipitate that forms of the nucleic acid is centrifuged and the supernatant is discarded. The pellet which contains the nucleic acid is taken up in 70 % ethanol in a subsequent step, centrifuged again, the supernatant is discarded and, after drying the pellet, it is resuspended in a desired buffer. However, the isopropanol/ethanol precipitation method is only practical for applications in a laboratory where relatively small volumes are used.

Apart from the limitation to small volumes, the described isopropanol/ethanol precipitation method has other serious disadvantages. Thus for reasons of operational safety and environmental protection it is very unfavourable to use the required isopropanol/ethanol volumes on an industrial process scale.



A method for isolating and purifying nucleic acids for use in gene therapy is described in WO 95/21177 in which the purification is essentially by centrifugation, filtration, an affinity chromatography or a chromatography on an inorganic chromatographic material and a chromatography on an ion exchanger. In order to remove endotoxins the nucleic acid is treated with an endotoxin removal buffer which contains 10 % Triton® X100 and 40 mmol/l MOPS buffer (3-morpholino-1-propane sulfonate). A disadvantage of this method is that the nucleic acid purified in this manner is contaminated with the pharmacologically unsafe substances Triton® and MOPS. In addition, although it is possible to deplete endotoxins to a content of ca. 100 EU/mg DNA (QIAGEN News 1-96, 3-5), a more extensive removal of endotoxins is not possible. However, nucleic acid preparations with an even higher purity are required for a therapeutic application, like for example a gene therapy, which are as free as possible of all impurities (in particular substantially free of endotoxins). Above all the endotoxin content of plasmid DNA preparations has been a hitherto unsolved problem as for example described by Cotten et al., Gene Therapy 1 (1994) 239 - 246.

K.-G. Wahlund and A. Litzén (Journal of Chromatography, 461 (1989), 73-87; 476 (1989), 413-421) describe a method named "field flow fractionation (FFF)" suitable for analytical and micropreparative applications for separating protein mixtures and plasmids according to their respective molecular weights. As in a cross-flow filtration the approach flow on the ultrafiltration membrane is tangential but, in contrast to cross-flow filtration, the separation is based on the different migration of the molecules in the stream of carrier fluid. Hence the elution of the molecules to be

the membranes to obtain a purified or/and concentrated plasmid DNA solution.

It has now been found that nucleic acid solutions can be purified and concentrated with the method of the present invention using a cross-flow filtration system. In this connection a surprising and new feature is that the nucleic acids are not damaged by the cross-flow filtration (CFF). Previously it has always been assumed that the shear forces occurring in the CFF would lead to damage of nucleic acids in particular to strand breaks. Therefore CFF was previously only used to concentrate and diafiltrate proteins. In addition the method of the invention not only enables nucleic acids to be obtained in large amounts and of a desired purity, but the method of the present invention also avoids the use of organic solvents which is a major advantage toxicologically as well as with regard to safety and environmental aspects.



membranes made of polyether sulfone (PES), modified PES, polyvinylidene difluoride (PVDF), cellulose triacetate or regenerated cellulose. Hollow fibre coil modules are also suitable for the method according to the invention. Membranes with an exclusion size of 1 - 1000 kilodalton (kD) are preferably used, 10 - 300 kD is more preferred and 10 - 100 kD is most preferred. The endotoxin depletion factor (ratio of endotoxin content of the nucleic acid preparation before cross-flow filtration to the endotoxin content of the nucleic acid solution after cross-flow filtration) that is achieved in the present invention is at least 10 : 1, preferably at least 200 : 1. The endotoxin content of the solution is very low after cross-flow filtration and is preferably < 0.1 EU/mg nucleic acid. The nucleic acids obtained in the present invention are essentially undamaged and essentially have no single-strand or double-strand breaks.

In particular a plasmid DNA purified according to the invention exhibits only one dominant band after gel electrophoretic separation which corresponds to the "covalently closed circle" conformation. Furthermore, apart from small amounts of the open circle and linearized circle conformations, no other bands are present.



Legend:

1% agarose gel

- Lane 1: DNA length standard II (Boehringer Mannheim GmbH; Cat. No. 236250)
 - Lane 2: DNA length standard III (Boehringer Mannheim GmbH, Cat. No. 528552).
 - Lane 3: pBR322 (Boehringer Mannheim GmbH, Cat. No. 481238) (0.4 μ g)
 - Lane 4: pCMV-CAT after CFF, 0.19 μ g (bulk active substance solution)
 - Lane 5: pCMV-CAT after CFF, 0.45 μ g (bulk active substance solution)
 - Lane 6: pCMV-CAT after CFF, 0.71 μ g (bulk active substance solution)
 - Lane 7: TE buffer
 - Lane 8: pBR322 (Boehringer Mannheim GmbH, Cat. No. 481238) (0.4 μ g)
 - Lane 9: DNA length standard III (Boehringer Mannheim GmbH; Cat. No. 528552)
 - Lane 10: DNA length standard II (Boehringer Mannheim GmbH, Cat. No. 236250).
-

The claims defining the invention are as follows:

1. Method for purifying or/and concentrating plasmid DNA in a solution,
wherein
the solution containing plasmid DNA is guided tangentially past one or several semi-permeable membranes in a process that proceeds continuously such that the plasmid DNA molecules are retained by the membranes and substances with a lower molecular weight can pass through the membranes to obtain a purified or/and concentrated plasmid DNA solution.
2. Method as claimed in claim 1,
wherein
the nucleic acid has a size of ≥ 150 base pairs.
3. Method as claimed in one of the previous claims,
wherein
a solution with a volume of 1 - 10,000 l is processed.
4. Method as claimed in one of the previous claims,
wherein
a solution with a volume of 1 - 100 l is processed.
5. Method as claimed in one of the previous claims,
wherein
the solution is guided past the membrane(s) under pressure whereby the cross-flow pressure is larger than the transmembrane pressure.



6. Method as claimed in claim 5,
wherein
a transmembrane pressure of 0.2 - 3.0 bar is used.
7. Method as claimed in one of the previous claims,
wherein
a retentate flow rate of 100 - 4000 l/h·m² is used.
8. Methods for purifying and/or concentrating plasmid DNA in a solution,
substantially as hereinbefore described with reference to the Examples.

DATED this 31st day of March, 2000

ROCHE DIAGNOSTICS GmbH

By its Patent Attorneys

DAVIES COLLISON CAVE



Figure 1



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